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C5a Receptor Oligomerization

II. FLUORESCENCE RESONANCE ENERGY TRANSFER STUDIES OF A HUMAN G PROTEIN-COUPLED RECEPTOR EXPRESSED IN YEAST*

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Recent studies demonstrate that members of the superfamily of G protein-coupled receptors (GPCRs) form oligomers both *in vitro* and *in vivo*. The mechanisms by which GPCRs oligomerize and the roles of accessory proteins in this process are not well understood. We used disulfide-trapping experiments to show that C5a receptors, expressed in mammalian cells, reside in membranes as oligomers (Klco, J. M., Lassere, T. B., and Baranski, T. J. (2003) *J. Biol. Chem.* 278, 35345–35353). To begin to address how C5a receptors form oligomers, we now use fluorescence resonance energy transfer experiments on human C5a receptors expressed in the lower eukaryote *Saccharomyces cerevisiae*. C5a receptors tagged with variants of the green fluorescent protein display energy transfer in intact yeast, demonstrating that mammalian accessory proteins are not required for C5a receptor oligomerization. In both intact yeast cells and membrane preparations, agonist does not affect FRET efficiency, and little energy transfer is observed between the C5a receptor and a co-expressed yeast pheromone receptor (encoded by *STE2*), indicating that C5a receptor oligomerization is both receptor-specific and constitutive. FRET studies performed on fractionated membranes demonstrate similar levels of energy transfer between tagged C5a receptors in endoplasmic reticulum compared with plasma membrane, and urea washing of membranes has little effect on the extent of energy transfer. The oligomerization of C5a receptors expressed in yeast displays characteristics similar to those observed for other GPCRs studied in mammalian cells. This model system should prove useful for further studies to define mechanisms of oligomerization of mammalian GPCRs.

G protein-coupled receptors (GPCRs)[1] are a diverse superfamily of receptors that mediate numerous physiological effects in response to sensory, hormonal, and neurotransmitter stimuli (1). There is mounting evidence that GPCRs can associate as homo- and/or hetero-oligomers in the plasma membrane, perhaps with functional consequences (2, 3). *In vitro* studies of many GPCRs in all major families (rhodopsin-like, secretin, and metabotropic glutamate) demonstrate that receptors can be co-immunoprecipitated as differentially epitope-tagged homodimers in the case of the opioid, Ig-Hepta, and calcium-sensing and metabotropic glutamate-5 receptors (4–8). Disulfide-trapping studies have mapped potential oligomer interfaces in dopamine D2 receptors (9) and the C5a receptor (46). The availability of variants of the green fluorescent protein has enabled *in vivo* studies of GPCRs. Fluorescence resonance and bioluminescence resonance energy transfer experiments (FRET and BRET, respectively) provide evidence of oligomerization of many GPCRs; the β_2-adrenergic receptor and the chemokine receptor CCR5 have been shown to homodimerize with BRET, the δ-opioid receptor has been shown to dimerize with BRET and time-resolved FRET, and the yeast pheromone receptor has been shown to oligomerize with FRET in live yeast (10–13). Oligomers of GPCRs have recently been visualized; elegant atomic force microscopy studies of native disc membranes reveal that rhodopsin packs in the membrane as rows of dimers (14).

Whereas the physical association of GPCRs into oligomers has been well described, the mechanisms that assemble GPCRs into higher ordered structures and the roles of membrane scaffolds and accessory proteins in regulating receptor oligomerization remain poorly understood. Several studies demonstrate that receptor oligomers may occur early in their biosynthesis; for example, the metabotropic GABA B1 receptor is retained in the endoplasmic reticulum unless the GABA B2 subunit is co-expressed (15). In addition, energy transfer studies demonstrate that the mammalian CCR5 receptor (11) and the yeast pheromone receptors (16) form constitutive oligomers in the endoplasmic reticulum, suggesting that this phenomenon might be essential for proper receptor assembly, both in yeast and mammalian cells. For the majority of GPCRs, it is unclear if the association of receptors into oligomers is a receptor-autonomous process or if accessory proteins mediate specific associations between homo- and heterodimers. Proteins that interact with receptors to regulate their internalization and desensitization might also be involved in regulating GPCR oligomerization at the plasma membrane; examples include the heterotrimeric G proteins, arrestins, caveolins, clathrin, and adaptor proteins (17). What roles these interactions play in

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; FRET, fluorescence resonance energy transfer; BRET, bioluminescence resonance energy transfer; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; C5aR, C5a receptor; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; PIPES, 1,4-piperazinediethanesulfonic acid.

receptor physiology and how they might affect attempts to study GPCRs in mammalian cells remains unknown.

In the current study, we take advantage of the ability to express the C5a receptor in yeast to begin to explore the mechanisms of receptor oligomerization. Yeasts are an established system for studying mammalian GPCR structure and function and offer several advantages. Many mammalian GPCRs (including the C5a, somatostatin, adenosine, and muscarinic receptors) can functionally couple to the yeast mating response pathway through the yeast Gα protein, Gpa1, or engineered G proteins that contain portions of both Gpa1 and human G proteins (18–22). Yeast strains that lack endogenous mating factor receptors can be easily engineered, thus providing a receptor-null environment in which to study mammalian receptors. Moreover, yeast pheromone receptors tagged differentially with cyan fluorescent protein and yellow fluorescent proteins have been shown to homo-oligomerize in living yeast cell populations (13, 16), suggesting that yeasts are an appropriate system for studying oligomerization of mammalian GPCRs. However, since haploid *S. cerevisiae* express a single type of GPCR, either the α- or a-factor receptor (encoded by either *STE2* or *STE3*, respectively), it was unclear whether yeasts have the ability to fold and assemble other GPCRs into specific oligomeric structures. Yeasts lack some known accessory proteins that might act as scaffolds to mediate GPCR oligomerization in a mammalian system (*e.g.* typical arrestins and caveolins), although they do contain an actin cytoskeleton, and yeast membrane proteins do undergo clathrin-mediated endocytosis (23).

In this FRET study, we asked whether or not the C5a receptor, a mammalian GPCR that forms oligomers in mammalian cells (46), similarly forms oligomers when expressed in the lower eukaryote *S. cerevisiae*. We reasoned that if C5a receptors in yeast did not form oligomers, as assessed by FRET, then mammalian proteins might be required for the assembly of higher order receptor complexes. If this were the case, one could also conclude that oligomerization is not required for the activation of G proteins, since C5a receptors can activate the yeast pheromone response pathway. Instead, our experiments demonstrate that C5a receptors tagged with CFP or YFP display energy transfer, both in intact yeast and in urea-washed membrane preparations. These results provide support for a model of receptor oligomerization whereby the association of monomers occurs as a receptor-autonomous process, without the aid of mammalian scaffold proteins or other peripheral membrane proteins.

EXPERIMENTAL PROCEDURES

Construction of Receptor Plasmids—Plasmids encoding truncated yeast pheromone (Ste2) receptors lacking 30 carboxyl-terminal residues and fused at the COOH-terminal end to genes for cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were as described (16, 24). Plasmids encoding the wild-type, full-length C5a receptor fused at the COOH-terminal end to either YFP (pBN741) or CFP (pBN744) were made using pBN433 or pBN482, wild-type C5a receptor plasmids (18). YFP and CFP templates with 5′ and 3′ sequences homologous to pBN433 were generated via PCR using the following oligonucleotides: 5′-ACG CGC TCC ACA GTG GAC ACT ATG GCC CAG AAG ACC CAG GCA GTG ATG AGT AAA GGA GAA GAA C-3′ and 5′-CTA CGC GAT CAT GGC GAC CAC ACC CGT CCT GTG GAT CGC TCT AGA TTA TTT GTA TAG TTC ATC-3′. pBN433 was linearized with *Xba*I, and homologous recombination was carried out in yeast strain BY1142 (see "Yeast Strains"). Receptor sequences were confirmed by sequencing at the recombination fusion region. Using standard molecular techniques, the resulting YFP-containing plasmid, pBN721, was used to generate pBN741 (C5a receptor tagged with YFP) from pBN482. Two untagged C5a receptor-encoding plasmids, pBN433 and pBN482, were used as controls (16). For experiments testing the function of tagged C5a receptor, we used the following plasmids: pBN 741, C5aR-YFP-encoding plasmid; pBN482, wild-type C5a receptor-encoding plasmid; pBN444, prepro-α fused to C5a ligand-encoding plasmid; and pBN443, prepro-α-encoding plasmid (18).

Yeast Strains—Yeast strain BY1142 (18) (*MATα far1Δ1442 tbt1-1 FUS1-HIS3 can1 ste14::trp1::LYS2 ste3Δ1156 gpa1(41)-Gα$_{i2}$ lys2 ura3 leu2 trp1 his3 ade2*) was transformed by electroporation and plated on medium lacking leucine and adenine. This strain was used for experiments to test the ability of yeast coexpressing C5aR-YFP and C5a ligand to grow in the absence of histidine and in the presence of the competitive inhibitor of the *HIS3* gene product, 3-aminotriazole. BY1142 was also used in all experiments measuring FRET between co-expressed tagged C5a receptors. All experiments using α-factor receptor-encoding plasmids utilized *ste2Δ* yeast strain BY1184 (*MATa ADE2 his3Δ1 leu2-3, 112 LYS2 MET15 trp1 ura3-52 sst1Δ5(bar1Δ) ste2Δ*) (16). BY1184 was transformed by the lithium acetate method and plated on selective medium. Colonies were selected and grown in liquid cultures for fluorescence resonance energy transfer experiments. For assays testing receptor function, we used yeast strain BY1173 (MATα *his3 leu2 trp1 ura3 can1 gpa1Δ::ADE2::3XHA far1Δ::URA3Δ fus1::FUS1-HIS3 LEU2::FUS1-lacZ sst2Δ::URA3Δ ste2Δ::G418R trp1::GPA1/Gα$_{i1}$*) made by integrating the Gpa1/Gα$_{i1}$ construct (21) at the *TRP1* locus of the yeast chromosome.

Fluorescence Resonance Energy Transfer—Experiments were carried out using a PerkinElmer LS50B luminescence spectrophotometer and two different Spex Fluoromax instruments, with consistent results for all instruments used. Experiments were performed as previously described (13, 16, 25). Briefly, 3 ml of overnight yeast cell cultures (A_{600} = 0.3–0.5) were washed three times with 3 ml of 100 mM Tris-Cl, pH 7.4, and harvested by centrifugation. The cultures were transferred to glass or plastic cuvettes and excited at 440 nm (CFP λ_{max}). An emission scan was collected between 460 and 610 nm at 0.5 or 2 nm/s, depending on the instrument used. In all cases, a blank generated from scans of yeast expressing two untagged receptor plasmids (on separate nutritional markers) was subtracted from experimental and YFP and CFP control scans to control for background autofluorescence of yeast. All cuvettes including the blank were first corrected for cell number by removing yeast and adding back either more concentrated yeast or buffer and aligning the spectra at 610 nm, where emission by either fluorescent protein is minimal. In experiments using ligand, C064 was added to the 3-ml cuvettes to a final concentration of 1 μM after initial data were collected in the absence of ligand, and scans were collected at three time points (1, 15, and 60 min after the addition of ligand).

Apparent FRET efficiency was calculated as previously described (13, 16). Control CFP and YFP emission peaks (excitation 440 nm) were normalized and then subtracted from the CFP plus YFP emission peak to yield a YFP emission spectrum due solely to FRET. The peak area of this emission spectrum was then divided by the peak area of an emission spectrum generated by directly exciting YFP at its λ_{max}, 490 nm.

Subcellular Fractionation—Subcellular fractionation of yeast cells was carried out by equilibrium density gradient centrifugation as previously described (13). Briefly, 200-ml yeast populations were grown to an A_{600} of 0.5, and then 2 ml of 1 M NaN_3 was added, and the cells were placed on ice for 15 min. Cells were harvested by centrifugation and then washed and harvested again in 50 ml of sorbitol buffer (10 mM Tris-Cl, pH 7.6, 0.8 M sorbitol, 10 mM NaN_3, 1 mM EDTA, pH 8.0. The cells were washed and resuspended in 1 ml of sorbitol buffer and then in 1 ml of 10% sucrose buffer (10 mM Tris-Cl, pH 7.6, 1 mM EDTA, 10% sucrose). Cells were then harvested and resuspended in 1 ml of 10% sucrose buffer with protease inhibitors (100 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, and 20 μM tosyl-phenylalanine-chloromethyl ketone). Glass beads were added for mechanical disruption, and the cells were vortexed vigorously more than seven times for 30-s intervals. Unbroken cells were removed by centrifugation at 300 × *g* for 5 min. 0.5 ml of lysate was mixed with 0.5 ml of 50% sucrose buffer and applied to a linear gradient of 40, 50, 60, and 68% sucrose buffers.

Membranes were spun for 24 h at 150,000 × *g* in an ultracentrifuge in a Sorvall SW50.1 swinging bucket rotor. For each preparation, 13 (350-μl) fractions were collected from the top of the gradient and assayed via Western blot using antibodies to the following yeast marker proteins: mouse monoclonal antibodies specific for Vph1 (vacuolar) and Dpm1 (endoplasmic reticulum) (Molecular Probes, Inc., Eugene, OR), 1:1000; Pma1 (plasma membrane), 1:5000, from Duane Jenness (26, 27); and Gda1 (Golgi) (C. Hirschberg, University of Massachusetts, Amherst, MA), 1:250 (28). 8–10 μl of each fraction was loaded per lane. Antibodies for C5a receptor (raised against an amino-terminal peptide) and green fluorescent protein (Santa Cruz Biotechnology) (to distinguish between the Ste2 and C5a receptors) were also used. Fractions 1–4 and 5–9 were pooled, and each set was diluted to 3 ml in 100 mM Tris acetate, pH 8.0, 500 mM potassium acetate, 1 mM magnesium

TABLE I

Growth of yeast co-expressing wild-type or YFP-tagged C5a receptor and full-length C5a ligand on 3-aminotriazole

Yeast strain BY1142, which contains a *FUS1-HIS3* gene fusion, was transformed with separate high copy plasmids as indicated and plated on media lacking uracil and adenine. Resulting colonies were then replica-plated onto media lacking uracil and histidine and containing various concentrations of 3-aminotriazole (0–10 mM), as indicated below, and scored as follows: +++, 75–100% of colonies from the original plate grew on the media; ++, 50–75%; +, 25–50%; +/−, 25% or less; −, no growth. WT, wild-type.

Receptor/Ligand	[3-aminotriazole]					
	0 mM	1 mM	2 mM	5 mM	10 mM	*n*
WT C5aR (pBN482) + C5a (pBN444)	+++	++	++	+	+/−	4
C5aR-YFP (pBN741) + C5a (pBN444)	+++	++	++	+	+/−	4
WT C5aR (pBN482) − C5a (pBN443)	+++	−	−	−	−	2
C5aR-YFP (pBN741) − C5a (pBN443)	+++	−	−	−	−	2

acetate, 0.1 mM EDTA, a buffer in which agonist binding affinity is sensitive to GTPγS (29). In experiments using C064 ligand in membranes, GTPγS was added to the buffer to a final concentration of 50 μM. Western blots were done after each experiment to confirm plasma membrane enrichment.

Total Yeast Membranes—For experiments using total yeast membrane preparations, yeast cells were harvested and lysed as above. The crude lysate was loaded in total onto a 1.5-ml preparation of sucrose cushion (500 mM sucrose, 100 mM Tris acetate, pH 8.0, 1 mM EDTA) and spun for 30 min at 217,000 × *g* in a Sorvall 100-AT6 rotor). After harvesting, the membrane pellet was resuspended in a sucrose cushion, and an energy transfer experiment was carried out. Some membranes were treated with 6 M urea in 10 mM Tris-Cl, pH 8.0, and 1 mM EDTA with protease inhibitors after this initial spin. Membranes were resuspended in the 6 M urea solution and incubated for 30 min at 4 °C. Then the preparation was spun at 217,000 × *g* for 15 min using the same rotor and centrifuge. The urea wash was repeated, and then the membranes were resuspended in a sucrose cushion.

β-Galactosidase Assays—To assess the functionality of our fluorescent protein-tagged C5a receptors as well as receptor mutants, we utilized a yeast strain containing a chromosomally integrated *FUS1-lacZ* reporter gene. Yeasts were grown to confluence overnight in minimal medium and then diluted to 0.15 OD in medium 90 μl of yeast suspension was added to each well in a 96-well plate. Cells were exposed to C064 peptide at a concentration of 1 μM for 4 h and then lysed with chlorophenol red-β-D-galactopyranoside lysis substrate buffer (50/50 mixture of chlorophenol red-β-D-galactopyranoside, 4.86 mg/ml, in 25 mM PIPES, pH 6.8, and 5% Triton X-100 in 250 mM PIPES, pH 6.8). Plates were incubated for 1 h at 37 °C, and conversion of chlorophenol red-β-D-galactopyranoside to chlorophenol red was measured by measuring the absorbance of light at 570 nm.

Fluorescence Microscopy—Fluorescence and Nomarski images of yeast cells expressing wild-type C5a receptors tagged with YFP were obtained using a DAGE cooled CCD camera mounted on an Olympus BH-2 microscope equipped with a DPLanApo100UV × 100 objective.

RESULTS

CFP- and YFP-tagged C5a Receptors Are Functional in Yeast—The physiological role of the human C5a receptor is to mediate neutrophil chemotaxis toward the 74-amino acid polypeptide C5a. C5a contains a globular core domain with several basic amino acids and an unstructured carboxyl terminus (30). When the core domain of C5a docks with the acidic amino acids on the C5a receptor amino terminus, the ligand's unstructured carboxyl terminus can then interact with a second binding site in the core of the receptor and activate the receptor (31–34). An engineered hexapeptide ligand called C064 or ChaCha, F-K-P-dChaCha-dR (where Cha represents cyclohexylalanine) (32), mimics the actions of the C5a COOH terminus to activate the C5a receptor. For our studies, we used the C064 peptide to activate C5a receptors, since the C5a ligand is too large to traverse the yeast cell wall. Previous work demonstrates that fusion to green fluorescent protein does not disrupt C5a receptor function; when C5aR-green fluorescent protein is expressed in neutrophils, the receptors bind ligand with normal affinity, and the cells migrate chemotactically to a gradient of C5a (35).

We tagged full-length C5a receptors with either YFP or CFP. To ensure that the fusion proteins functioned well in yeast, we characterized C5aR-CFP/YFP in two assays. First, we used a growth assay to test for the ability of wild-type C5aR and C5aR-YFP to respond to C5a ligand. In this assay, receptor activation of G proteins results in the expression of a *FUS1-HIS3* reporter gene, thus allowing yeast to grow on histidine-deficient plates. The extent of activation can be assessed by the ability of yeast to grow on plates containing increasing concentrations of 3-aminotriazole, a competitive inhibitor of the *HIS3* gene product. Both the wild-type C5aR and the C5aR-YFP signal at levels that support growth on 5 mM 3-aminotriazole (Table I), whereas neither the wild-type C5aR nor the C5aR-YFP displays significant signaling in the absence of ligand. Second, we performed dose-response experiments with intact yeast. We expressed the tagged C5a receptors (pBN741 and pBN744) in yeast strain BY1173, which contains a chromosomally integrated Gpa1/$G\alpha_{i1}$ chimeric G protein and an integrated *FUS1-lacZ* reporter gene (21) and tested their signaling in response to C064 ligand. The tagged receptors were functional at a level similar to that of untagged C5a receptors in yeast (Fig. 1). In addition, the EC_{50} for C064 activation of C5a receptors (~50 nM) is comparable with the EC_{50} of C064 activation of endogenous C5a receptors in neutrophils (20 nM) (32). Using standard fluorescence microscopy techniques, the majority of C5aR-YFP was found to localize to the plasma membrane and endoplasmic reticulum (Fig. 2). The localization of the C5aR is similar to the localization of Ste2Δtail-YFP. Relative to the truncated pheromone receptor, more of C5aR-YFP was observed in punctate, mobile structures likely to be endosomes in yeast (the COOH-terminal deletion in the pheromone receptor prevents the receptor from undergoing endocytosis (13, 16)). Expression levels of the tagged and untagged forms of the C5a receptors in yeast were similar, as seen by Western blotting.[2]

C5a Receptors Demonstrate FRET in Living Yeast—To determine whether oligomerization of human C5a receptors occurs in yeast, as has been reported for the pheromone receptor (13, 16), we performed FRET experiments. We co-expressed C5aR-CFP (pBN744) and C5aR-YFP (pBN741) in a standard yeast strain (BY1142). The apparent FRET efficiency of tagged C5a receptors in living yeast populations was 12.6 ± 3.0% (n = 7) (Fig. 3*A*). This FRET efficiency is comparable with that of episome-expressed Ste2Δtail-YFP in living yeast populations (11.5 ± 2.2%, $n > 20$), which have consistently been shown to oligomerize in yeast (13, 16).

To test whether oligomerization of C5a receptors in yeast was receptor-specific, we co-expressed tagged C5aR-YFP and Ste2Δtail-CFP and performed FRET experiments in living yeast. The apparent FRET efficiency was 2.9 ± 1.9% (n = 8) (Fig. 3*B*), suggesting that C5a receptors and pheromone receptors do not oligomerize to a significant degree. We cannot rule out some interaction between C5a and α-factor receptors, since the FRET efficiency could be affected by packing interactions

[2] D. Floyd and T. Baranski, unpublished data.

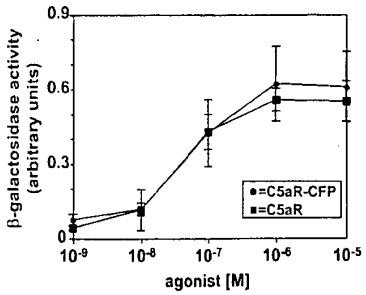

FIG. 1. **β-Galactosidase activity of wild-type and YFP-tagged human C5a receptors expressed in yeast.** Human C5a receptors with and without contiguous YFP constructs were expressed episomally in yeast containing an integrated *FUS1-lacZ* gene reporter. The hexapeptide C064 ligand was added to an assay mix at the indicated concentrations, and activation of the mitogen-activated protein kinase pathway was quantitated using colorimetry.

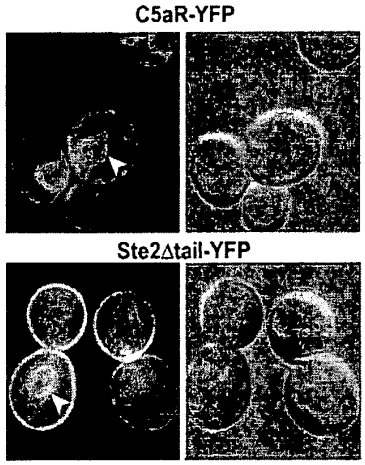

FIG. 2. **Subcellular localization patterns of YFP-tagged C5a and Ste2Δtail receptors in yeast.** Wild-type C5aR and truncated Ste2 receptors were tagged at their intracellular carboxyl termini with YFP and examined by fluorescence and differential interference contrast microscopy. YFP-tagged C5a receptors (*top panels*) were expressed on high copy plasmids. YFP-tagged α-factor receptors (*bottom panels*) were expressed from their endogenous promoter on single copy plasmids for fluorescence microscopy experiments but were expressed on high copy plasmids for all other experiments. α-Factor receptors were expressed in cells that carried a deletion of the chromosomal α-factor receptor gene. The *arrowheads* indicate perinuclear localization of YFP-tagged proteins in the endoplasmic reticulum.

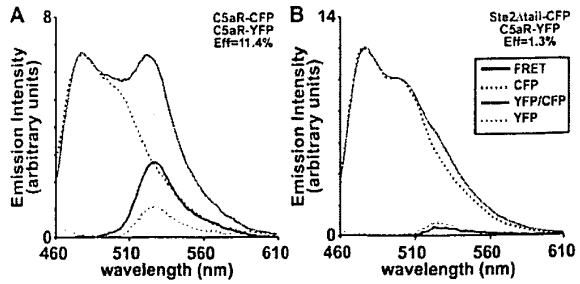

FIG. 3. **Use of FRET to detect oligomerization *in vivo* of wild-type C5a receptors and lack of oligomerization between C5a and Ste2Δtail receptors.** The indicated CFP- and YFP-tagged receptors were co-expressed for FRET experiments. FRET data were collected and analyzed as described under "Experimental Procedures." *Panels* in this *figure* and similar subsequent *figures* show four emission spectra obtained upon excitation of cells at the λ_{max} for CFP as follows: one from cells co-expressing the indicated CFP- and YFP-tagged receptors (*gray solid line*); a second from cells expressing only the indicated CFP-tagged receptor (*black dotted line*); a third from cells expressing only the indicated YFP-tagged receptor (*gray dotted line*); and a fourth that shows the fluorescence emission due specifically to FRET (*black solid line*) from cells co-expressing the indicated CFP- and YFP- tagged receptors. Emission due to FRET was calculated by subtracting the second and third emission curves from the first emission curve. Ste2Δtail receptors were expressed in cells that carried a deletion of the chromosomal *STE2* gene. Efficiencies of FRET (*Eff*) obtained with CFP- and YFP-tagged receptors are shown. The spectra and indicated FRET efficiencies are the results of a representative experiment; the average values and S.D. from multiple determinations are reported under "Results."

that alter the orientation of one fluorophore relative to the other. Also, it is unknown what the sensitivity of these FRET assays is for detecting protein-protein interactions; nonetheless, the low FRET efficiency observed between C5aR-YFP and Ste2Δtail-CFP is similar to that reported for the yeast glucose transporter Hxt1-YFP and Ste2Δtail-CFP, which do not oligomerize in yeast (16). The apparent FRET efficiency measured between α-factor and C5a receptor is much lower than that for either homophilic interaction (2.9% *versus* 11.5 and 12.6%, respectively; $p < 0.001$ for both). The expression levels of α-factor and C5a receptors in our system are similar (Fig. 5*A*); thus, it is unlikely that differences in protein expression lead to the decrease in FRET efficiency between α-factor and C5a receptor.

C5a Receptor FRET Is Unaffected by Agonist in Living Yeast—Work by other investigators has demonstrated very different effects of ligand binding on the amount of GPCR oligomerization, either increasing, decreasing, or no change, depending on the type of receptor (10, 36). To test whether the C5a receptor oligomers are affected by ligand activation of the receptor, we performed FRET experiments in the presence of C064, a hexapeptide agonist of the C5a receptor. Wild-type C5aR-C5aR FRET experiments were done before and after the addition of C064 (final concentration of 1 μM). Emission scans were collected 1, 15, and 60 min after ligand addition ($n = 3$ for each time point). There was no statistically significant difference in the apparent FRET efficiency after ligand addition (Fig. 4 and Table II). These results demonstrate that C5a receptors form constitutive oligomers, when expressed in yeast, and are consistent with the results obtained with disulfide-trapping experiments in endogenously expressed C5a receptors in neutrophils (46).

FRET between C5a Receptors Occurs on the Yeast Plasma Membrane as Well as in Other Subcellular Compartments—To investigate where in yeast cells C5a receptors form oligomers, we performed subcellular fractionation of yeast expressing both C5aR-CFP and C5aR-YFP and yeast expressing control plasmids and then performed FRET experiments with pooled fractions. Fractions 1–4 were enriched in Golgi, endoplasmic reticulum, and vacuole proteins (not shown), whereas fractions 5–9 generally represented plasma membrane-enriched fractions (Fig. 5*A*). FRET occurred between differentially tagged C5a receptors in both pooled fractions (Fig. 5, *B* and *C*). The FRET efficiency in plasma membrane-enriched fractions (5–9) was 18.3 ± 5.2% ($n = 7$), which is similar to the FRET efficiency of tagged C5a receptors in the endoplasmic reticulum and Golgi-enriched fractions (1–4), which was 13.2 ± 4.0% ($n = 3$). These data support the hypothesis that C5a receptors form oligomers early in their biosynthesis and membrane targeting.

C5a Receptor and α-Factor Receptor Do Not Demonstrate FRET in Yeast Organelles or the Plasma Membrane—We hypothesized that the absence of FRET between fluorescently tagged α-factor and C5a receptors was due to a lack of interaction between the receptors and not because the receptors were localized in different membrane compartments. To differentiate between these possibilities, we performed FRET experiments on membranes subjected to sucrose gradient fraction-

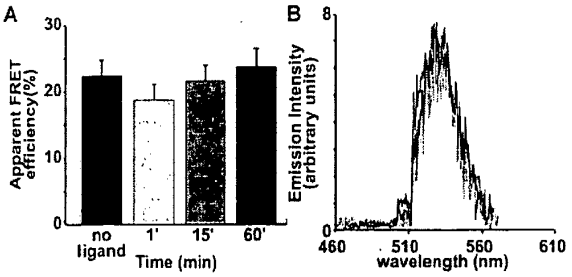

FIG. 4. **The effect of ligand addition upon C5a receptor FRET efficiency.** The FRET efficiency and S.D. of a representative C5a receptor FRET experiment are shown before and after the addition of C064 ligand *in vivo*. *A*, a comparison of FRET efficiencies before and at various time points after ligand addition from a representative experiment. *B*, the comparison of FRET spectra for the representative C5a receptor FRET experiment before and after the addition of C064 ligand, with each spectrum *shaded* as in its respective *bar* in *A*.

TABLE II

Effect of C064 ligand on FRET in whole cells: Effect of C064 ligand and GTPγS upon FRET in membrane fractions

The ability of C5a receptors to self-associate in the absence and presence of ligand in whole cells and in the absence and presence of ligand and GTPγS in the indicated membrane fractions was quantified by calculating the apparent FRET efficiency as described under "Experimental Procedures."

FRET assay type	Average FRET efficiency	S.D. (±)	*p* value	*n*
Whole Cells, C5aR-C5aR				
−C064	24.4	4.8		3
+C064 (1 min)	22.0	6.1	0.60	3
+C064 (15 min)	25.5	4.7	0.71	3
+C064 (60 min)	25.5	2.3	0.70	3
Fractionated Membranes, C5aR fractions 5–9 and 10–13				
−C064 and GTPγS	18.9	4.0		3
+C064 and GTPγS	18.9	3.3	0.73	3

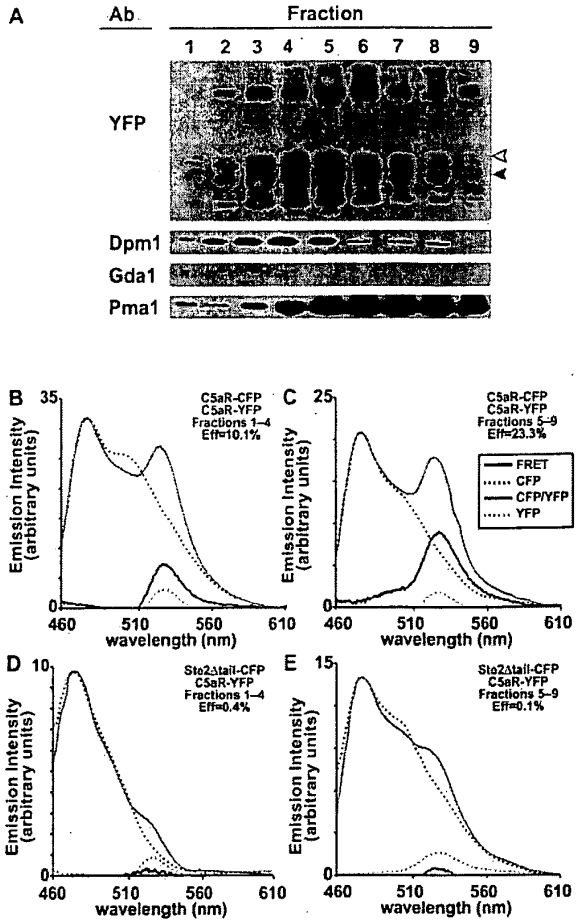

FIG. 5. **Subcellular fractionation of C5a and Ste2Δtail receptors.** Cell extracts were prepared and fractionated by sucrose density gradient centrifugation. *A*, fractionation of intracellular organelles across the gradient (fraction 1 (*top*) and fraction 9 (*bottom*)) was assessed by immunoblotting with antibodies (*Ab*) specific for marker proteins: Gda1 (Golgi), Dpm1 (endoplasmic reticulum), and Pma1 (plasma membrane (*PM*)). C5a receptor-YFP fusions appear as a 51-kDa band (*open arrowhead*), and Ste2Δtail-CFP fusions appear as a 47-kDa band (*closed arrowhead*) and a band representing a smaller proteolytic fragment. CFP- and YFP-tagged forms of C5a receptors were co-expressed, or tagged C5a and Ste2Δtail receptors were co-expressed on separate high copy plasmids in appropriate yeast strains as under "Experimental Procedures." *B–E*, the indicated fraction pools were analyzed by performing FRET experiments as described under "Experimental Procedures." Efficiencies of FRET obtained with CFP- and YFP-tagged receptors in the indicated gradient fraction pools are shown. The indicated FRET efficiencies are the results of one representative experiment.

ation of yeast expressing differentially tagged C5a receptors and α-factor receptors. Both the C5a and α-factor receptors were localized at similar levels in the endoplasmic reticulum and at the plasma membrane, as shown by Western blotting (Fig. 5*A*). The C5a receptors and the pheromone receptors migrate as monomers (*filled* and *open arrowhead*, respectively) and as an SDS-resistant dimer band. We were not able to establish lysis conditions that quantitatively solubilized either C5a or α factor receptors. The apparent FRET efficiency between pheromone receptors tagged with CFP and C5a receptors tagged with YFP was low and similar to values obtained in whole cells. FRET efficiency in fractions 1–4 (enriched in endoplasmic reticulum) was 0.5 ± 0.6% (n = 4), and efficiency in fractions 5–9 (enriched in plasma membrane) was 1.4 ± 1.8% (n = 3) (Fig. 5, *D* and *E*). We cannot rule out that the receptors reside in separate microdomains of cellular membranes; indeed, this might be one way that cells might cluster receptors into signaling complexes in the membrane. Nevertheless, these data further support the claim that C5a receptors oligomerize to the exclusion of other GPCRs in the endoplasmic reticulum and plasma membrane in yeast.

Biochemical Characterization of FRET of GPCRs in Yeast Membrane Fractions—To investigate further the extent to which the ligand or G protein could affect GPCR oligomers, we performed FRET assays with yeast plasma membrane fractions and then added C064 ligand and GTPγS with mixing (37), collected scans (at 1 min after addition), and compared the FRET peaks. Activation of the Gα subunit of the G protein in the presence of 1 μM nonhydrolyzable GTPγS dissociates G protein from receptor. There was no difference between the apparent FRET efficiency of C5a receptors before and after activation of the G protein; the average efficiency in untreated fractions (5–9) was 18.9%, and in treated fraction pools it was 18.9% (n = 3) (Fig. 6*A* and Table II). These results support the hypothesis that C5a receptors form constitutive oligomers and that G proteins do not significantly affect the extent of oligomerization.

Since the mechanism of GPCR oligomerization is not well understood, we undertook a set of experiments to remove peripheral membrane proteins that might mediate GPCR oligomerization in yeast. We performed FRET assays using crude yeast membranes that were washed with 6 M urea to denature peripheral membrane proteins. The membranes were then resuspended in sucrose buffer and analyzed for FRET. There was a small but significant decrease in FRET between untreated and treated membranes co-expressing tagged C5a receptors (untreated membranes, 15.2 ± 4.3%; treated membranes,

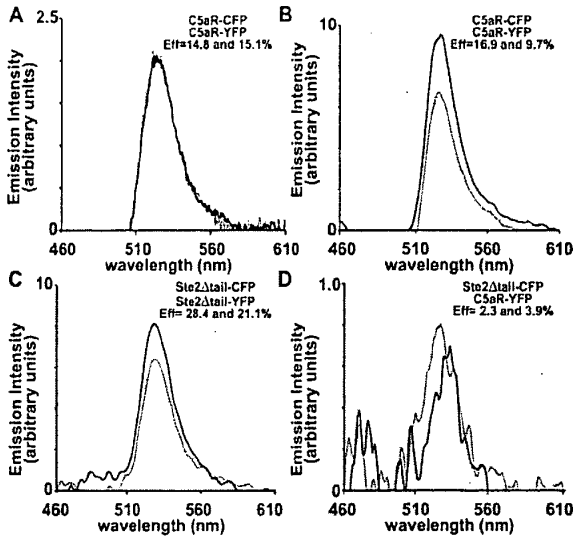

FIG. 6. **Effect of ligand, GTPγS, and urea stripping on FRET of C5a receptors and Ste2Δtail receptors.** CFP- and YFP-tagged forms of C5a receptors, Ste2Δtail receptors, or C5a and Ste2Δtail receptors were co-expressed on separate high copy plasmids in appropriate yeast strains as described under "Experimental Procedures." *A*, the cell extracts were fractionated by sucrose density gradient centrifugation. Fractions 5–9, which were enriched in plasma membrane, were analyzed by performing FRET experiments as described under "Experimental Procedures." Then 1 μM C064 hexapeptide ligand and 50 μM GTPγS were added to the buffer of the C5aR-CFP and -YFP co-expressing membranes, and FRET scans were collected again. The emission scans due solely to FRET are shown here (before (*black line*) and after (*gray line*)), and the FRET efficiencies given are for the before and after scans for this representative experiment. *B–D*, the indicated receptors were co-expressed, and crude membrane extracts were prepared as described under "Experimental Procedures." FRET experiments were conducted before and after washing the membrane preparations twice in 6 M urea. Emission scans due solely to FRET are shown here (before (*black line*) and after (*gray line*)), and the FRET efficiencies given are for this representative experiment.

10.6 ± 2.4%, $p = 0.022$) (Fig. 6*B*, Table III). Experiments performed using yeast expressing α-factor receptors showed a similar decrease in FRET between tagged α-factor receptors, with an average efficiency of 35.9 ± 6.9% in untreated membranes and an average efficiency of 28.5 ± 5.7% in treated membranes, $p = 0.018$ (Fig. 6*C*, Table III). The slight decrease in observed FRET might be explained by two possibilities. Peripherally associated membrane proteins (in either plasma membrane or endoplasmic reticulum) might have a small effect upon the oligomerization of both C5a receptors and α-factor receptors. Alternatively, urea treatment could directly affect the ability of the CFP and YFP to serve as donors and acceptors for energy transfer. In either event, both the C5a receptor and the pheromone receptor exhibit significant FRET, despite urea washing of total membranes. Of note, urea treatment did not significantly increase the apparent FRET between co-expressed α-factor and C5a receptors (Fig. 6*D*, Table III), demonstrating that peripheral membrane proteins do not appear to actively segregate pheromone receptors away from C5a receptors.

TABLE III
Effect of urea treatment on FRET in crude membranes

C5a and Ste2Δtail receptor FRET was assessed before and after washing membranes with 6 M urea and was quantified by calculating the apparent FRET efficiency as described under "Experimental Procedures."

Crude membranes	C5aR-C5aR	Ste2Δtail-Ste2Δtail	Ste2Δtail-C5aR
Average FRET efficiency (− urea) (%)	15.2	35.9	1.7
S.D. (− urea)	4.3	6.7	.9
Average FRET efficiency (+ urea) (%)	10.6	28.5	2.9
S.D. (+ urea)	2.4	5.7	1.3
p value	0.022	0.019	0.146
n	3	4	2

DISCUSSION

Using FRET analysis as a measure of receptor association, we have shown that the human C5a receptor forms oligomers *in vivo* when expressed in the yeast *S. cerevisiae*. The oligomerization of C5a receptors is unaffected by ligand or G protein and occurs without the aid of mammalian proteins that might have an accessory role during oligomerization. We have also shown that this oligomerization is specific and occurs both in the endoplasmic reticulum and at the plasma membrane. These results are supported by previous studies of pheromone receptor oligomerization in yeast (13, 16) and are consistent with biochemical evidence for C5a receptor oligomerization in mammalian cells (46).

In addition, these studies represent the first energy transfer experiments using mammalian GPCRs expressed in yeast cell populations. Our conclusions are likely to be applicable to the GPCR oligomerization field in general. In several important aspects, the human C5a receptor in our FRET experiments in yeast demonstrates very similar properties to those described for other GPCRs in mammalian cells. Bouvier and co-workers (10), in some of the initial studies using energy transfer between tagged receptors in living cells, demonstrated that β_2-adrenergic receptor formed constitutive oligomers, and activation with isoproterenol had only a small effect on levels of dimerization. In further studies, they also demonstrated constitutive oligomers for CCR5 receptors (11) as well as constitutive homo- and hetero-oligomers between oxytocin and vasopressin receptors (38). Interestingly, subcellular fractionation studies of cells expressing CCR5 receptors revealed that BRET occurred to a similar extent between receptors in endoplasmic reticulum as at the plasma membrane, leading the authors to conclude that oligomerization of the CCR5 occurs early in the biosynthesis of the receptor (11). Similar results were obtained for pheromone receptors in yeast (16) and for the C5a receptor in this study, suggesting that initial oligomerization of GPCRs in the endoplasmic reticulum might represent a general requirement for the proper assembly and intracellular targeting of receptors.

Studies on other GPCRs provide different conclusions regarding the effect of ligand on oligomerization. FRET analysis of somatostatin receptors demonstrated that activation by ligand induces somatostatin receptor dimerization, both homo- and heterodimerization with other members of the somatostatin receptor family (39, 40). In contrast, the ligand-activated B(2) bradykinin receptor, endogenously expressed in PC-12 cells, showed decreased amounts of dimerization compared with unactivated receptor, as measured by cross-linking and Western blotting (41). Also, Devi and co-workers reported that the δ-opioid receptor demonstrated lower levels of dimerization in the presence of ligand, as assayed by cross-linking and co-immunoprecipitation in stably transfected Chinese hamster ovary cells (4). In contrast, Milligan and co-workers, using time-resolved FRET and BRET, found that the human δ-opioid receptor displays constitutive oligomerization at the cell surface, which is not regulated by receptor occupancy (12). Some of the apparent inconsistencies between these studies might be explained by different sensitivities of the techniques employed

and the ability of the approaches to discriminate between changes in dimerization *versus* changes in higher order oligomerization.

A fundamental question for understanding GPCRs is how receptors form oligomers. Our research directly addresses this question and is one of the first studies to do so. The ability to express a human GPCR in yeast allows us to test three simple models for how receptors oligomerize: 1) self-assembly of receptors into dimer or higher order oligomers; 2) association of receptors via interactions with common protein or lipid structures; or 3) tethering of receptors mediated by scaffold proteins adapted to interact with a limited number of receptors. In the first model, monomeric receptors assemble into dimers or higher order structures in a receptor-autonomous process. The specificity of the interaction would therefore be dictated by the complementarity of binding surfaces at the intermolecular interfaces. If this model were correct, one would predict that C5a receptors would form oligomers with C5a receptors but not with pheromone receptors, as was observed.

In the second model, GPCRs are brought together by virtue of interactions with common cellular structures (*e.g.* actin cytoskeleton, heterotrimeric G proteins, lipid rafts, and endocytic vesicles). Some of these structures are present in yeast, such as heterotrimeric G proteins, clathrin, adaptor proteins, and lipid rafts (42). However, the yeast genome does not encode other candidate proteins, such as GPCR kinases and caveolins, which might mediate C5a receptor interactions. If this second model is correct, there are two potential outcomes. If C5a receptors display energy transfer in yeast through interactions with orthologous yeast structures, then one would predict that C5a receptors would associate with pheromone receptors, unless the C5a receptors interact with different structures than the yeast receptors, which seems unlikely. If mammalian proteins or lipids are required for interactions between receptor monomers, then no FRET should be observed for the C5a receptor expressed in yeast.

In the third model, the oligomerization of C5a receptors observed in neutrophil membranes occurs through interactions with specific scaffold proteins. A yeast two-hybrid analysis, using the carboxyl tail of the C5a receptor as bait, identified Wiskott-Aldrich syndrome protein. Given its ability to regulate actin dynamics, Wiskott-Aldrich syndrome protein might play a role in connecting the C5a receptor to the actin cytoskeleton at the leading edge of neutrophils as they undergo chemotaxis (43). Other studies, also using the carboxyl tails of GPCRs as bait for yeast two-hybrid analyses, have identified an increasing number of interacting proteins for GPCRs (17). Many of these scaffold proteins bind GPCRs through specific PDZ domains; for example, the Na^+-H^+ exchanger regulatory factor protein was identified as a specific interacting protein for the β_2-adrenergic receptor (44). It is unlikely that yeast would express any proteins capable of specifically tethering C5a receptors to one another. Therefore, the third model predicts that C5a receptors would not form oligomers when expressed in yeast.

These models are not mutually exclusive. For example, GPCRs could form dimers in an autonomous process in the endoplasmic reticulum (first model), whereas at the plasma membrane, receptor dimers are clustered in discrete signaling complexes (second and third models). Overall, the results of FRET analysis of C5a receptor and pheromone receptors in yeast provide support for the first model of receptor oligomerization that proposes that GPCRs self-assemble into dimers or higher order oligomers as they are synthesized and transit through the secretory pathway.

Based on our previous genetic studies of the structure and function of the transmembrane segments of the C5a receptor expressed in yeast, we hypothesized that the C5a receptor would form oligomers when expressed in yeast. In those studies using saturation mutagenesis, we performed a comprehensive analysis on each of the seven transmembrane segments of the C5a receptor and found that amino acids in the second and fourth transmembrane segments were essential for the function of the C5a receptor despite the fact that their side chains point outward from the center of the helix bundle and into the lipid membrane (45). We postulated that these functionally important residues might interact either at a dimer interface or with another membrane scaffold protein or lipid raft. Our current results, using FRET in yeast cells, demonstrate that oligomerization of the human C5a receptor does occur in yeast and is both receptor-specific and ligand-independent. The conservation of functional residues at potential oligomer interfaces suggests that oligomerization is a prerequisite for the ability to activate G proteins. If monomeric GPCRs activated G proteins, then there would not be selective pressure for mutant C5a receptors to conserve these residues. An important caveat to this argument is that the oligomer interface might be essential for GPCRs to properly fold in the endoplasmic reticulum. In this scenario, monomeric receptors might be capable of activating G proteins, provided they could assemble and be transported to the plasma membrane.

The saturation mutagenesis studies of the C5a receptor in yeast complement the recent disulfide-trapping studies of the D2 dopamine receptor, which implicate helix 4 as the dimer interface (9). In the companion paper by Klco *et al.* (46), we describe disulfide-trapping studies with C5a receptors in mammalian membranes. These studies demonstrate that cysteines placed in intracellular loops 1 and 2 and the carboxyl terminus, but not in intracellular loop 3, efficiently form disulfide bonds when exposed to ambient oxygen and cupric orthophenanthroline. No single model of symmetric dimers can adequately account for these findings, suggesting higher order oligomers of C5a receptors. Whereas the FRET data on C5a receptors in yeast do not directly address the question of dimer/oligomer interfaces, these results demonstrate that yeasts are likely to be a useful model system in which to perform mutagenesis studies to further define the oligomerization domains of mammalian GPCRs.

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